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Glutathione Transferase Activities toward Herbicides Used Selectively in Soybean*

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Abstract: Using extracts from suspension-cultured cells of soybean (*Glycine max* cv. Mandarin) as a source of active enzymes, the activities of glutathione transferases (GSTs) catalysing the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and selective herbicides were determined to be in the order CDNB ≫ fomesafen > metolachlor = acifluorfen > chlorimuron-ethyl. GST activities showed a thiol dependence in a substrate-specific manner. Thus, GST activities toward acifluorfen and fomesafen were greater when homoglutathione (hGSH), the endogenously occurring thiol in soybean, was used as the co-substrate rather than glutathione (GSH). Compared with GSH, hGSH addition either reduced or had no effect on GST activities toward other substrates. In the absence of enzyme, the rates of hGSH conjugation with acifluorfen, chlorimuron-ethyl and fomesafen were negligible, suggesting that rapid hGSH conjugation in soybean must be catalysed by GSTs. GST activities were subsequently determined in 14-day-old plants of soybean and a number of annual grass and broadleaf weeds. GST activities of the plants were then related to observed sensitivities to post-emergence applications of the four herbicides. When enzyme activity was expressed on a mg^{-1} protein basis, all grass weeds and *Abutilon theophrasti* contained considerably higher GST activity toward CDNB than soybean. With fomesafen as the substrate, GST activities were determined to be in the order soybean ≫ *Echinochloa crus-galli* > *Digitaria sanguinalis* > *Sorghum halepense* = *Setaria faberi* with none of the broadleaf weeds showing any activity. This order related well to the observed selectivity of fomesafen, with the exception of *A. theophrasti*, which was partially tolerant to the herbicide. Using metolachlor as the substrate the order of the GST activities was soybean > *A. theophrasti* ≫ *S. halepense* > *Amaranthus retroflexus* > *Ipomoea hederacea*, with the remaining species showing no activity. GST activities toward metolachlor correlated well with the selectivity of the herbicide toward the broadleaf weeds but not toward the grass weeds. Acifluorfen and chlorimuron-ethyl were selectively active on these species, but GST activities toward these herbicides could not be detected in crude extracts from whole plants.

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1 INTRODUCTION

The relative rate of herbicide degradation in crops and competing weeds is a major determinant of herbicide selectivity.[1] In soybean (*Glycine max* (L.) Merr.) several chloroacetanilide herbicides,[2] acifluorfen,[3] chlorimuron-ethyl[4] and fomesafen[5] are all rapidly detoxified by conjugation with homoglutathione (hGSH, γ-L-glutamyl-L-cysteinyl-β-alanine) as shown in Fig. 1. The triazinone herbicide metribuzin also undergoes hGSH conjugation in this species following its sulphoxidation.[6] In each case this detoxification has been implicated as a major factor in determining the tolerance of soybean to these herbicides.[2–5] In soybean, hGSH is used in the conjugation of toxic electrophilic xenobiotics rather than the more commonly observed glutathione (GSH, γ-glutamyl-L-cysteinyl-L-glycine).[2–7] This is because hGSH represents 99% of the available low-molecular-weight thiol in the leaves of this species.[7,8]

The rate of herbicide detoxification by conjugation with GSH or hGSH in plants is determined by a number of factors. These include the relative reactivity of the herbicide, the bioavailability of the thiol co-substrate, the presence of appropriate glutathione transferases (GSTs, EC 2.5.1.18) and sequestration and catabolism of the resulting conjugate.[9] Recent studies with atrazine, alachlor, metolachlor and fluorodifen in seedlings of maize and associated weeds suggested that GST activities toward each herbicide correlated well with the relative rates of detoxification by GSH conjugation and with herbicide tolerance in the individual species.[1] The availability of GSH appeared less important.[1] In view of their importance in herbicide selectivity the GSTs of maize have now been described at both the molecular[10] and biochemical level with five distinct isoenzymes determined.[11–13]

In contrast, very little is known regarding the importance of GST's in the herbicide tolerance of soybean. This is despite the literature showing that several herbicides undergo rapid detoxification by conjugation with hGSH.[2–7] A study by Breaux *et al.* suggested that the selectivity of chloroacetanilide herbicides in soybean could be partially explained by the higher concentrations of hGSH in the crop relative to the lower levels of GSH in competing weeds, but the role of GSTs was not reported.[7] Subsequently, GST activities toward

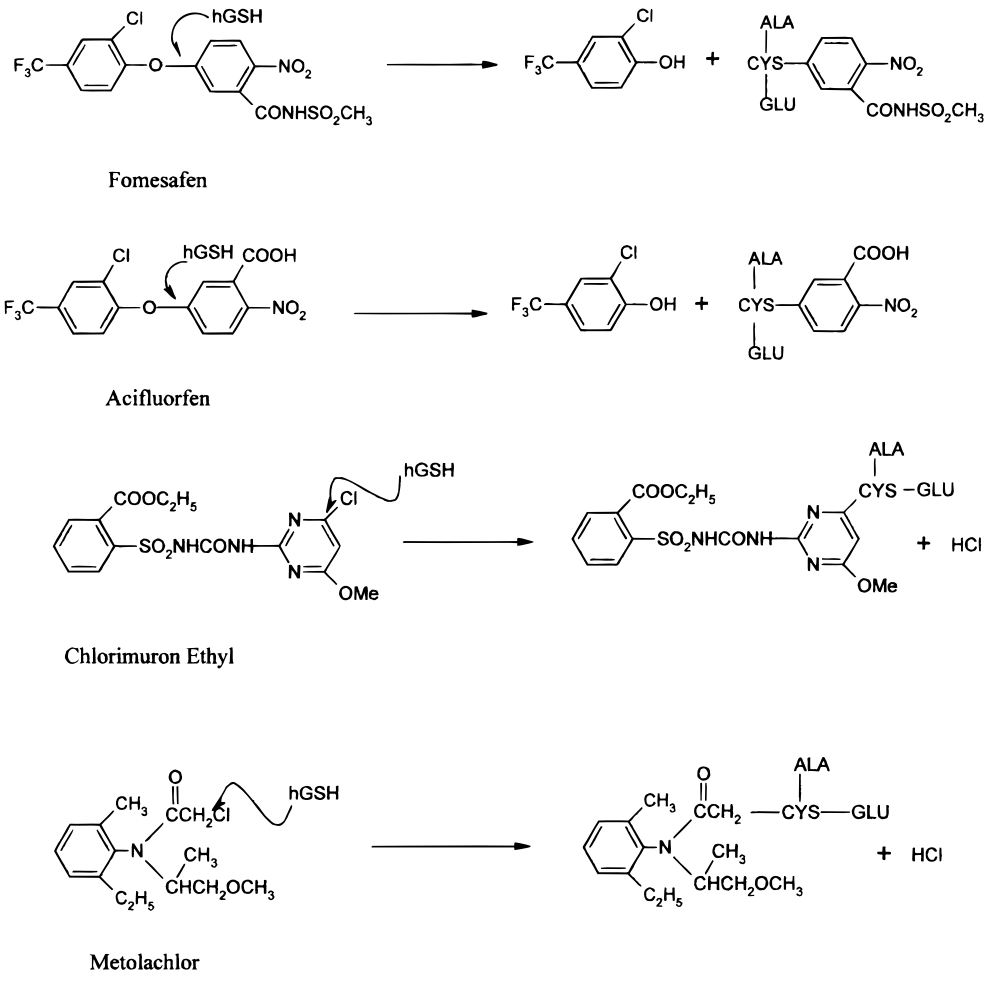

Fig. 1. The detoxification of soybean selective herbicides by conjugation with homoglutathione (hGSH). In each case the site of nucleophilic attack by hGSH is indicated with an arrow.

1-chloro-2,4-dinitrobenzene (CDNB)[14,15] and metolachlor[15] have been determined in soybean seedlings exposed to heat-shock[14] and 2,4-dichlorophenoxyacetic acid.[15] However, the existence of GST activities toward the selective herbicides acifluorfen, fomesafen and chlorimuron-ethyl have only been inferred from the rapid hGSH-mediated detoxification the herbicides undergo in plants[3–5] and the enzyme activities have not been demonstrated in soybean *in vitro*. To rectify this situation we now report on the GST activities toward a number of selective herbicides in plants and cell cultures of soybean and in competing weeds. These results have then been correlated with the sensitivities of these species to the herbicides.

2 MATERIALS AND METHODS

2.1 Plant material

Seeds of soybean cv. ICI 297 were supplied by Zeneca Agrochemicals and seeds of *Setaria faberi* Herrm., *Echinochloa crus-galli* (L.) Beauv., *Digitaria sanguinalis* (L.) Scop., *Sorghum halepense* (L.) Moench., *Abutilon theophrasti* Medic., *Ipomoea hederacea* Gray. and *Amaranthus retroflexus* (L.) by Herbiseed Ltd, Wokingham, UK. For the determination of enzyme activities, seeds were sown in Levington Universal compost and grown in a controlled environment chamber at 25°C under a 16 h photoperiod at a light intensity of 140 μmol m^{-2} s^{-1} using cool white fluorescent lighting. At 14 days after sowing, the plants were uprooted and washed free of contaminating soil, then blotted dry, weighed and frozen in liquid nitrogen. Tissue was stored at −80°C until required. Suspension-cultured cells of soybean (cv. Mandarin) were obtained from Zeneca Agrochemicals and maintained on an orbital shaker (120 rev min^{-1}) in darkness at 28°C using Gamborg B5 minimal medium supplemented with 20 g $litre^{-1}$ sucrose and 1 mg $litre^{-1}$ 2,4-dichlorophenoxyacetic acid. Suspension cultures were sub-cultured, with a 20% inoculum by volume, every 10 days and when required the cells were harvested on nylon mesh filters under vacuum, weighed and frozen in liquid nitrogen.

2.2 Chemicals

Authentic hGSH was prepared by Zeneca Agrochemicals as described by Adang *et al.*[16] and references therein. Acifluorfen, chlorimuron-ethyl and metolachlor, all of >99% purity, were obtained from Greyhound Chem. Service Inc., Birkenhead, Merseyside L43 4XF. Fomesafen was supplied by Zeneca Agrochemicals. A reference GSH conjugate of metolachlor was prepared as detailed previously.[1] The GSH conjugate of chlorimuron-ethyl and *S*-[3-(*N*-methanesulphonyl-carbonyl)-4-nitrophenyl]glutathione (GS-MSCNP) and *S*-(3-carboxy-4-nitrophenyl)glutathione (GS-CNP) derived from the GSH-mediated cleavage of the diphenyl ether bond of fomesafen and acifluorfen respectively were prepared by mixing an ethanol solution of the herbicide (10 mM; 1 ml) with sterile Tris HCl (0·1 M, pH 9; 9 ml) containing 10 mM GSH. The mixture was then incubated under aseptic conditions for five days at room temperature. After removing unreacted herbicide by partitioning with 2 volumes of ethyl acetate, a sample of the aqueous phase was applied to a silica-coated TLC plate containing a fluorescent indicator (Merck, Darmstadt, Germany). The plate was then developed in butan-1-ol + acetic acid + water (4 + 1 + 1 by volume) and the GSH conjugates identified from their UV absorbance and positive reaction with ninhydrin. The remainder of the sample was then applied to TLC plates and the respective GSH conjugates recovered in methanol and then concentrated to dryness. After confirming the purity of the conjugates by HPLC as described in Section 2.3, samples of the GSH conjugate of chlorimuron-ethyl and GS-MSCNP were analysed by fast atom bombardment mass spectrometry (FAB-MS). The spectra of the GSH conjugate of chlorimuron ethyl and MSCNP revealed parent mass ions (M + H) of *m/e* 686 and *m/e* 563 respectively, together with mass ions corresponding to the associated Na salts[4] which were consistent with the expected structures. The authentic GSH conjugates were then used to confirm the identity of the reaction products formed in the GST assays with the herbicides.

2.3 Determination of GST activities

Frozen plant tissue was homogenised in a solution of Tris HCl (0·1 M; pH 7·5), EDTA (2 mM) dithiothreitol (1 mM) and polyvinylpolypyrrolidone (150 g kg^{-1} tissue) at a rate of 3 ml solution per gram of tissue using a pestle and mortar cooled on ice with acid-washed sand added as an abrasive. The slurry was filtered through a double thickness of muslin and then centrifuged (17 000*g*, 20 min, 4°C). The supernatant was decanted and adjusted to 80% saturation with respect to ammonium sulfate and the protein pellet collected by re-centrifugation. The protein pellet was dissolved in Tris HCl (20 mM, pH 7·5) containing dithiothreitol (1 mM) and desalted using the same buffer either by passage through a Sephadex G 25 column (Pharmacia PD 10) or by dialysis for 16 h. GST activity toward CDNB was determined spectrophotometrically.[13] Assays with the herbicides consisted of herbicide dissolved in either acetone (chlorimuron-ethyl and metolachlor) or methanol (fomesafen and acifluorfen) (10 mM; 10 μl) the enzyme extract (120 μl), GSH or hGSH (100 mM; 20 μl) and the relevant buffer (50 μl).

For assays with fomesafen, acifluorfen and chlorimuron-ethyl the buffer was 0.4 M Tris HCl pH 9·5 and for metolachlor 0·1 M potassium phosphate pH 6·8. In all cases the assays were initiated with the addition of GSH or hGSH and the samples incubated at 37°C for 1 h. After stopping the reaction with 5 μl of concentrated hydrochloric acid, the sample was frozen at −20°C and then allowed to thaw prior to clarification by centrifugation (11 000*g*, 2 min). A 50 μl sample of the supernatant was analysed by HPLC.[1] In all cases enzyme activities were expressed in katals, where 1 katal = 1 mole of reaction product formed s^{-1}, after correcting for product formation in the absence of enzyme. The protein contents of the extracts were determined using a dye-binding reagent with gamma-globulin as the reference protein as recommended by the manufacturer (Bio-Rad).

2.4 Herbicide selectivity studies

For post-emergence herbicide selectivity studies, soybean (cv. ICI 297) and the weed species were grown in John Innes potting compost No. 3 in a glasshouse for 14 days. When first treated with herbicides the plants were at the following growth stages and median heights: soybean two trifoliate leaves (32 cm), *A. theophrasti* two to three leaves (3 cm), *A. retroflexus* four to five leaves (4 cm) and *I. hederacea* two leaves (6 cm). For the grass weeds *D. sanguinalis* had five leaves and one to three tillers (7 cm), *E. crus-galli* four leaves, no tillers (8 cm), *S. faberi* three leaves and no or one tiller (6 cm) and *S. halapense* four leaves and no tillers (6 cm). The plants were then sprayed with herbicides formulated with a non-ionic surfactant and organic solvent, using a track sprayer fitted with a 8002E T-jet nozzle delivering an equivalent of 200 litre ha^{-1}. Each herbicide was used at four application rates 1, 2, 4 and 8 with the 1 × rates for each compound being acifluorfen 50 g ha^{-1}, chlorimuron-ethyl 6·25 g ha^{-1}, fomesafen 37·5 g ha^{-1} and metolachlor 250 g ha^{-1}. Plants were visually assessed for phytotoxic injury using a percentage scoring system at seven and 13 days after treatment, by comparison with plants sprayed with formulation alone.

3 RESULTS

3.1 GST activities in soybean

Before determining GST activities using herbicide substrates for which assays had not previously been described, it was first necessary to partially characterise the properties of the GSTs in plants and suspension cultures of soybean to determine a suitable source for further study. In initial studies with seedlings, a range of soybean cultivars were screened for GST activity toward CDNB. Extracts from cv. ICI 297 showed the highest GST specific activities (data not shown) and this cultivar was used for all subsequent plant studies. GST activities were then compared in various organs of five-day-old ICI 297 seedlings, in seeds and in soybean cell cultures in mid-growth phase (five days old). When CDNB was used as a substrate, GST specific activities were in the order: cell cultures (1·5 nkat mg^{-1}) > seeds (0·5 nkat mg^{-1}) > cotyledons (0·3 nkat mg^{-1}) > stem = root (0·2 nkat mg^{-1}). In view of the high GST activities determined, extracts from cell cultures were used to establish enzyme assays with the herbicides.

GST activities toward the herbicides were assayed using reversed-phase HPLC in order to separate the GSH and hGSH conjugates from the parent herbicides as previously described for metolachlor and other herbicides.[1] Examples of chromatograms showing the resolution of the hGSH conjugates from the herbicides acifluorfen, fomesafen and chlorimuron-ethyl are shown in Fig. 2. The elution profiles show several UV-absorbing peaks in addition to the substrate and reaction products. These peaks were due to natural products present in the soybean extracts and, with all analyses, enzyme extracts were incubated without GSH/hGSH to correct for co-chromatographing impurities. The elution profiles obtained with all three herbicides suggested that a significant proportion of the substrate had been converted to the respective conjugate. However, in all cases, only a minor proportion of the substrate had been metabolised, with a large proportion of the unchanged herbicide precipitating out of solution at the termination of the reaction and therefore not being injected onto the HPLC. In the absence of enzyme, the rates of formation of conjugates of acifluorfen, fomesafen and chlorimuron-ethyl were below the limit of detection of the assay, although an appreciable rate could be determined with metolachlor (Table 1). The non-enzymic conjugation rate with metolachlor was higher than reported previously,[1] due to the 10-fold

TABLE 1
Retention Times of the GSH and hGSH Conjugated Metabolites, including Rates of Formation in the Absence of Enzyme

Herbicide	*Thiol*	*Retention time (min)*	*Non-enzymic rate[a] (pkat)*
Acifluorfen	GSH	10·7	<0·011
	hGSH	11·2	<0·011
Chlorimuron-ethyl	GSH	19·5	<0·006
	hGSH	19·6	<0·006
Fomesafen	GSH	10·7	<0·012
	hGSH	11·1	<0·012
Metolachlor	GSH	18·5	1·180
	hGSH	18·7	1·083

[a] Non-enzymic rate determined at pH 6·8 for metolachlor and pH 9·5 for the other herbicides.

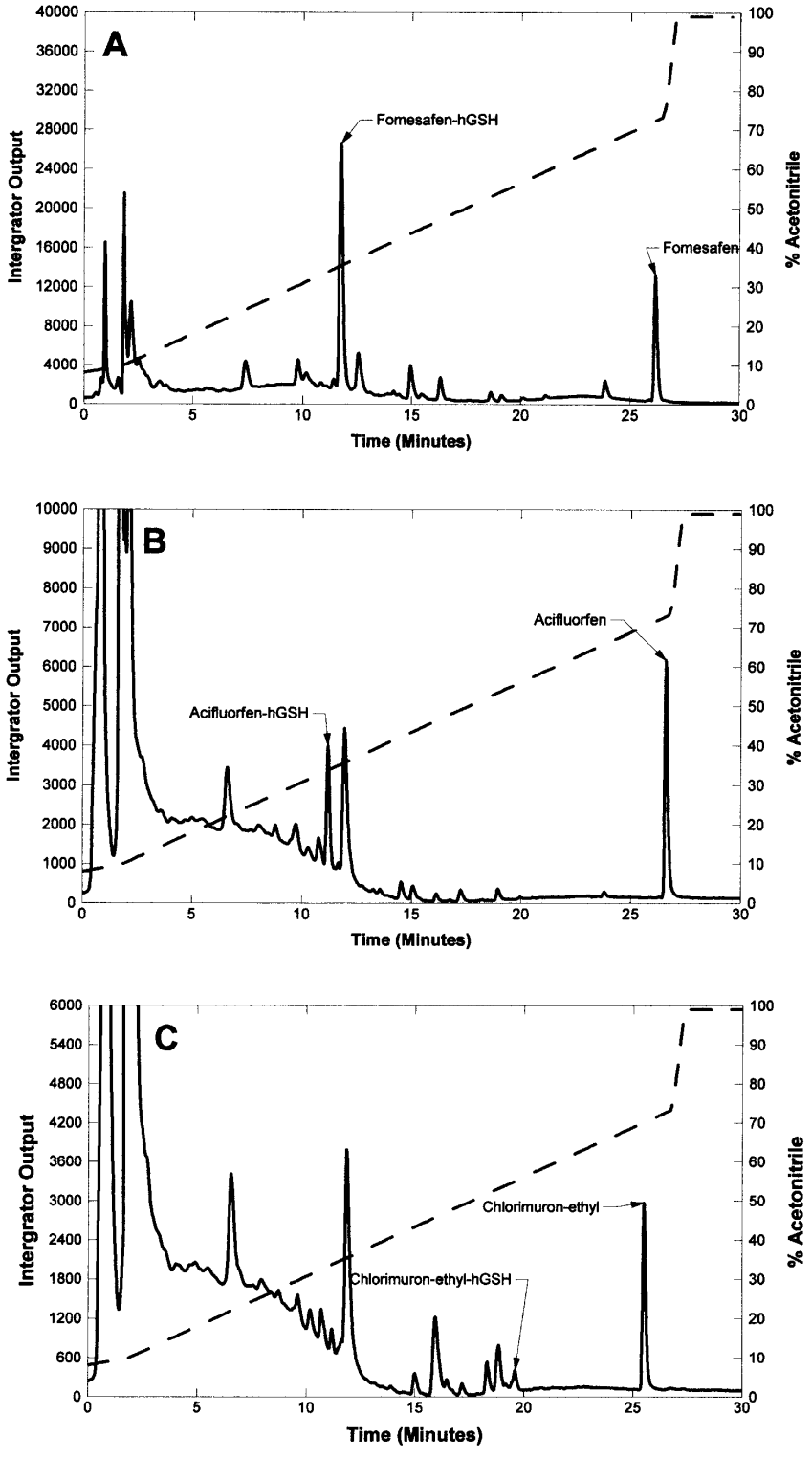

Fig. 2. HPLC analysis of hGSH conjugates formed following incubation of extracts from five-day-old soybean cell suspension cultures with hGSH and the herbicides A. fomesafen, B. acifluorfen and C. chlorimuron-ethyl. The *y*-axis on the left-hand side shows values used by the HPLC in the integration of the peaks and can be used to compare the relative amounts of conjugate formed under identical conditions. The eluting solvent gradient profile is indicated with the broken line.

higher concentration of GSH used in the assays in the current study. With chlorimuron-ethyl, the conjugate was quantified following a calibration with the authentic herbicide. Although the UV absorption spectra of chlorimuron-ethyl and its conjugate are known to differ,[4] the degree of error introduced by a calibration based on the parent herbicide was minimal. In quantifying GS-MSCNP and GS-CNP formed respectively from fomesafen and acifluorfen, it was assumed that, following cleavage of the ether bond, both fragments of the

molecule exhibited approximately half the molecular extinction coefficients of the intact herbicides.

The novel GST activities toward acifluorfen, fomesafen and chlorimuron-ethyl with GSH as co-substrate were partially characterised using extracts from soybean suspension cultures. In all cases the formation of the respective GSH conjugates was directly proportional to incubation time up to 60 min and to protein content in the range 0–2 mg protein per assay. Using tris HCl buffer, the pH optimum for all three activities was determined to be 9.5, which is similar to the optimum determined for the cleavage of fluorodifen.

Since soybean plants contain hGSH, rather than GSH, as the dominant low-molecular-weight thiol[8] it was important to determine whether the GSTs in soybean showed a preference for the thiol co-substrate. Following incubation of the enzyme with the herbicides in the presence of hGSH the corresponding hGSH conjugates formed were found to elute slightly later than the respective GSH conjugates (Table 1). Extracts from five-day-old cell cultures and 14-day-old seedlings were assayed for GST activities toward CDNB and the herbicides in the presence of either GSH or hGSH (Table 2). With the exception of metolachlor, all substrates were conjugated more rapidly in the extracts from cell cultures than in the extracts from the foliage of the seedlings. With acifluorfen and chlorimuron-ethyl as substrates GST activities could only be determined in the preparations from cell cultures. An interesting substrate-dependent preference for the type of thiol used in the conjugation reactions was identified. Acifluorfen and fomesafen were respectively conjugated four-fold and 14-fold more rapidly in the presence of hGSH than of GSH, in the extracts from cell cultures. In the case of fomesafen, this selectivity for the co-substrate was also observed in the extracts from seedlings, with GST activity being supported with hGSH but not with GSH. Conversely, in extracts from both the cell cultures and seedlings, GST activity toward CDNB was better supported by GSH than hGSH. In the cell cultures, GST activity toward metolachlor was also lower with hGSH than with GSH, but the GST activity in seedlings conjugated metolachlor equally rapidly with either thiol. GST activity toward chlorimuron-ethyl in the cell cultures was found to be equally low with either thiol co-substrate.

3.2 GST activities in soybean and competing weeds

To determine their likely contribution to herbicide detoxification and selectivity, GST activities toward CDNB, acifluorfen, chlorimuron-ethyl, fomesafen and metolachlor were determined in 14-day-old seedlings of soybean and associated weeds which have been described as problematical in North America.[17] In view of the importance of using the correct thiol co-substrate (Table 2) the GST activities of soybean were determined using hGSH. In the weed species GSH was used, as there is no evidence that plants other than legumes contain hGSH.[8]

GST activities toward chlorimuron-ethyl and acifluorfen could not be found in crude extracts from the foliage of any of the species. However, activity toward CDNB, fomesafen and metolachlor was measured in all species (Table 3). With the non-herbicide CDNB as substrate, the GST activities in weeds were appreciably greater than those of soybean except for *A. retroflexus* and *I. hederacea.* In contrast, soybean showed the highest GST-specific activity toward metolachlor and

TABLE 2
GST Activities in Extracts from the Foliage of 14-Day-Old Seedlings and Five-Day-Old-Cell Cultures of Soybean

		GST activity (pkat mg^{-1} protein)[a]	
Substrate	*Thiol*	*Cell cultures*	*Seedlings*
CDNB	GSH	1483 (±11)	260 (±70)
	hGSH	394 (±18)	68 (±9)
Acifluorfen	GSH	0·09 (±0·01)	ND[b]
	hGSH	0·35 (±0·02)	ND
Chlorimuron-ethyl	GSH	0·04 (±0·01)	ND
	hGSH	0·03 (±0·01)	ND
Fomesafen	GSH	0·18 (±0·01)	ND
	hGSH	2·57 (±0·02)	0·25 (±0·01)
Metolachlor	GSH	1·59 (±0·03)	6·26 (±0·11)
	hGSH	0·33 (±0·01)	6·17 (±0·05)

[a] Enzyme activity refers to the mean of independently prepared duplicates ± the variation from the mean after correcting for the non-enzymic conjugation rate.
[b] ND None detected.

TABLE 3
GST Activities toward CDNB and Herbicides in Extracts from Whole 14 Day-Old Plants of Soybean and Competing Weeds

Species	*GST activity (pkat mg^{-1} protein)*[a]		
	CDNB	*Fomesafen*	*Metolachlor*
Soybean	260 (±70)	0·25 (±0·03)	6·26 (±0·11)
A. theophrasti	520 (±160)	ND[b]	4·88 (±0·10)
A. retroflexus	140 (±30)	ND	0·08 (±0·03)
D. sanguinalis	580 (±40)	0·12 (±0·01)	ND
E. crus-galli	580 (±120)	0·15 (±0·01)	ND
I. hederacea	130 (±20)	ND	0·04 (±0·01)
S. faberi	1130 (±160)	0·11 (±0·00)	ND
S. halepense	1250 (±140)	0·10 (±0·01)	0·31 (±0·12)

[a] Values refer to the means of independent duplicate determinations ± the variation between the mean and the replicates.
The thiol used was GSH, except with soybean, when hGSH was added.
[b] ND None detected.

fomesafen. With metolachlor as substrate, only *A. theophrasti* showed comparable GST activity to the crop, all the other weeds having 10-fold lower activities, or less, than soybean. No GST activity toward fomesafen could be determined in *I. hederacea* or *A. retroflexus*. In *A. theophrasti* a UV-absorbing natural product present in the plant co-eluted with the GSH conjugate. Thus, although no activity could readily be determined, it was possible that this species showed basal GST activities toward fomesafen. All the grass weeds contained approximately half of the fomesafen-conjugating GST activity present in the crop.

3.3 Herbicide selectivity studies

In order to relate GST activities to herbicide selectivity, acifluorfen, chlorimuron-ethyl, fomesafen and metolachlor were each applied at four different application rates to soybean and the weeds of identical age to those used for assaying enzyme activities. Phytotoxicity was then assessed at seven and 13 days after treatment. To simplify presentation of the data, the results of the seven-day assessment at the lowest and highest rates of application used for each herbicide are shown in Table 4. Similar trends in herbicide tolerance were also obtained at 13 days and the data in Table 4 can therefore be considered to be representative.

Fomesafen was highly effective in controlling *A. retroflexus* and *I. hederacea*, causing intermediate injury to the remaining weed species and only minor injury to soybean. A similar spectrum of activity was seen with acifluorfen, though this herbicide was relatively more phytotoxic to soybean. Chlorimuron-ethyl was phytotoxic to all the broadleaf weeds but was less effective against the grasses, with little selectivity being shown at the lower dose rates toward either *E. crus-galli* or *D. sanguinalis*. Metolachlor is normally employed as a pre-emergence selective herbicide and therefore its post-

TABLE 4
Herbicide Selectivity in 14-Day-Old Plants of Soybean and Competing Weed Species

Species	*Application rate*	*Injury at seven days relative to controls* (%)			
		Fomesafen[a]	*Acifluorfen*[b]	*Chlorimuron-ethyl*[c]	*Metolachlor*[d]
Soybean	Low	10	38	5	5
	High	30	36	10	41
A. theophrasti	Low	40	62	79	6
	High	91	91	84	5
A. retroflexus	Low	93	100	85	51
	High	100	100	95	54
D. sanguinalis	Low	48	85	3	0
	High	76	93	0	31
E. crus-galli	Low	34	46	6	5
	High	59	62	65	46
I. hederacea	Low	80	90	60	23
	High	100	100	79	34
S. faberi	Low	40	73	16	39
	High	69	93	30	80
S. halepense	Low	41	94	20	20
	High	94	96	76	16

[a] Low application rate for fomesafen = 37·5 g ha^{-1}, high dose rate = 300 g ha^{-1}.
[b] Low application rate for acifluorfen = 50 g ha^{-1}, high dose rate = 400 g ha^{-1}.
[c] Low application rate for chlorimuron ethyl = 6·25 g ha^{-1}, high dose rate = 50 g ha^{-1}.
[d] Low application rate for metolachlor = 187·5 g ha^{-1}, high dose rate = 1500 g ha^{-1}.

emergence use in this study was atypical. At the lower application rates, metolachlor injured the weeds in the order *A. retroflexus* > *S. faberi* > *I. hederacea* > *S. halapense* but showed little selectivity toward the other species. *A. theophrasti* was tolerant to metolachlor at all application rates, whereas injury of soybean and some of the other weeds was greater at the higher rates.

4 DISCUSSION

Using an HPLC-based assay we have demonstrated that soybean cell cultures contain GSTs with activities toward the diphenyl ether, sulfonylurea and chloroacetanilide herbicides. Previously a GST with activity toward metolachlor was described in 2,4-dichlorophenoxyacetic acid-treated soybean seedlings,[15] but the presence of the other enzyme activities has only been inferred from metabolism studies. In soybean seedlings the hGSH-mediated detoxification of acifluorfen,[3] chlorimuron-ethyl[4] and fomesafen[5] is described as being extremely rapid and essentially complete within 24 h. In each case, the rapid hGSH-conjugation has been linked to the tolerance of the crop toward these herbicides. By determining the conjugation rate in the absence of any enzyme, our results suggest that acifluorfen, fomesafen and chlorimuron-ethyl are insufficiently electrophilic to undergo appreciable conjugation with hGSH in the absence of a GST to catalyse the reaction. It therefore follows that if conjugation with hGSH is a major determinant of the tolerance of soybean to these herbicides, then GST activity, rather than hGSH availability, would be the major mechanism of tolerance. This may not be the case with metolachlor, which underwent appreciable conjugation in the absence of any enzyme. Unfortunately, although GST activity toward acifluorfen, chlorimuron-ethyl and fomesafen could be determined in cell cultures, only the activity toward fomesafen could be measured in seedlings. Since the GST activity toward fomesafen in seedlings was 10-fold lower than in the cell cultures, it seems likely that the corresponding activities toward acifluorfen and chlorimuron-ethyl in the foliage were below the limit of detection of our HPLC-based assay. Alternatively, the GSTs active toward acifluorfen and chlorimuron-ethyl may be inactivated during their extraction from plants. In either event, although the GST activities toward chlorimuron-ethyl and acifluorfen cannot be determined in plants, our data would suggest that GSTs are required for the detoxification of these herbicides *in planta* as demonstrated for fomesafen and metolachlor.

The substrate-dependent variations in the preference for GSH and hGSH in the soybean GSTs demonstrated the importance of using the endogenously occurring thiol when assaying GSTs. The complexity in the extent to which the two thiols supported GST activity toward different substrates can be explained in two ways. Soybean may contain specific GSTs active toward fomesafen and acifluorfen which preferentially use hGSH, while additional GSTs with activities toward CDNB, chloroacetanilides and chlorimuron-ethyl use hGSH less efficiently. Alternatively, GSTs from soybean may be active toward a wide range of herbicides, as appears to be the case in maize,[11,13] but these activities are selectively supported by different thiols. Currently it is not possible to speculate which explanation is correct, especially as the utilisation of the two thiols by GSH/hGSH-requiring enzymes in plants is not well understood. Glutathione reductases from French bean (*Phaseolus vulgaris* L.) showed similar preferences for the oxidised forms of GSH and hGSH, while the enzyme in non-legume species, such as wheat and spinach, showed a preference for oxidised GSH.[18] In mammals, early studies reported that two GST isoenzymes from rat liver were able to use GSH and hGSH with equal efficiency.[19] However, later studies with purified GST isoenzymes from rat demonstrated that enzyme activities toward a variety of substrates were lower with hGSH than with GSH.[16]

Consistent with a key role for hGSH conjugation in the selectivity of fomesafen in soybean[5] the tolerant crop contained the highest GST activities toward the herbicide of all the species tested. In contrast, the two susceptible weeds, *A. retroflexus* and *I. hederacea* had no detectable GST activity toward fomesafen. Similarly the grass weeds *D. sanguinalis*, *S. halapense*, *S. faberi* and *E. crus-galli* showed intermediate sensitivities to fomesafen and all showed approximately half the GST specific activity toward the herbicide compared with soybean. An exception to the relationship between GSTs and fomesafen sensitivity was seen with *A. theophrasti*, which contained no detectable GST activity toward fomesafen *in vitro* but similar levels of herbicide tolerance to that determined in the grass weeds. This suggests that factors other than GSTs may mediate the intermediate tolerance of fomesafen in *A. theophrasti*.

With metolachlor, the relationship between GST activities and herbicide tolerance following post-emergence application was less clearcut. Tolerant soybean and *A. theophrasti* contained the highest GST activities toward metolachlor of any of the species tested, while sensitive *A. retroflexus* and *I. hederacea* contained 60-fold lower activities. Therefore, in the broadleaf species, GST activities could be correlated with herbicide tolerance. However, this was not the case with the grass weeds. Thus, *D. sanguinalis* and *E. crus-galli* showed similar tolerance to metolachlor to soybean and *A. theophrasti*, but contained no detectable GST activity toward the herbicide. Similarly, GST activity toward metolachlor could be determined in *S. faberi* which was sensitive, while *S. halapense* contained measurable GST activities toward metolachlor but was more sensitive to the herbicide than *D. sanguinalis* or *E. crus-galli*. Some of the results obtained with the weed

species differ from those obtained in a recent study, where low, but measurable, GST activities toward metolachlor were determined in *D. sanguinalis*, *E. crus-galli* and *S. faberi*.[1] Some of the variation results from differences in the concentration of GSH used in the two studies. Also, with *S. faberi* we have previously observed considerable variation in GST activities depending on the seed lots used,[20] which presumably arises from the genetic diversity present in *Setaria* species.[21] Our results demonstrate that such variation can also occur in other grass weeds and suggest that caution needs to be observed in defining herbicide tolerance mechanisms in genetically diverse populations.

Breaux *et al.*[7] suggested that the selectivity of chloroacetanilide herbicides, including metolachlor, in soybean and competing weeds could be explained by differences in the availability of hGSH and GSH in the crop and competing weeds. Thus, tolerant soybean was reported to contain 126 μg free thiol (GSH and hGSH) g^{-1} FW, while more susceptible weeds contained only 30–53% of this amount. In our study we did not determine the concentrations of available thiols in the different species. However, if the above values for GSH/hGSH are representative, it can be concluded that, as with the GST activities, only limited correlations between thiol availability and the sensitivity of weeds to post-emergence applications of metolachlor can be established.

Due to the absence of GST activity toward chlorimuron-ethyl and acifluorfen in soybean and the weeds, no conclusions can be drawn regarding the role of GSTs in the selectivity of these herbicides. With chlorimuron-ethyl, the studies of Brown and Neighbours[4] clearly demonstrated that tolerant soybean rapidly conjugated the herbicide with hGSH, while the susceptible *A. retroflexus* and *Xanthium pensylvanicum* Wallr. were able to detoxify the herbicide only slowly. Our studies suggest that a GST must be responsible for hGSH conjugation of chlorimuron-ethyl in soybean and one of the reasons for the elusive nature of this activity in plants may be the relatively low levels of GST-mediated catalysis required to detoxify such a low-application-rate herbicide. In any event it is also likely that factors such as uptake and target-site sensitivity influence the selectivity of chlorimuron-ethyl in soybean and weeds.[22] With acifluorfen, less is known regarding the relative importance of hGSH/GSH conjugation in determining selectivity, other than that the herbicide is rapidly detoxified in soybean in a manner analogous to fomesafen (Fig. 1).[3] In our studies, acifluorfen was more phytotoxic to soybean than fomesafen when applied at similar rates and this may be due, in part, to the low levels of acifluorfen-detoxifying GST activities in soybean.

In summary, our results show a broad correlation between the selectivity of fomesafen and the respective GST activities in soybean and competing weeds. No conclusions could be drawn regarding the importance of GSTs in the selectivity of acifluorfen or chlorimuron-ethyl as the activities could not be determined in crude extracts from any species. It will now be of interest to define the routes of metabolism of these herbicides in competing weeds in greater detail.

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